

# Immunocytochemically Defined Populations of Neurons Progressively Increase in Size Through Embryogenesis of *Hydra vulgaris*

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**Abstract.** The hydra nervous system shares many features with nervous systems of more complex organisms but serves as a unique model system due to its simplicity and constant regeneration. Development of neuron populations during and after hydra embryogenesis is not well understood. In this study, neurons were identified at prehatching and posthatching stages with RFamide or JD1 antisera. These populations were further subdivided into ganglion, sensory, or unclassifiable neurons, and all identified populations were statistically analyzed over developmental time. RFamide-positive neurons appeared 20 days after the cuticle formed around the embryo. The JD1-positive neuron population appeared just after hatching, but by adulthood it had surpassed the size of the RFamide-positive population. All neuron populations progressively increased through their adult levels. Density of most of the populations, however, did not. For instance, during the 5-fold increase in size that the hydra experienced between 5 days posthatching and adulthood, the number of RFamide-positive neurons rose approximately 2-fold and the number of JD1-positive neurons 4-fold. However, the density of neurons in each of these populations fell. These data do not support the hypothesis that large-scale culling of neurons during development, frequently found in other animals, occurs in hydra.

## Introduction

Cnidarian nervous systems are simple nervous systems with relatively few neurons and synapses (Grimmelikhuijzen *et al.*, 1988; Spencer, 1989, 1995). However, individual

cnidarian neurons and synapses exhibit many characteristics of more advanced systems. These commonalities include multiple immunochemically defined neuronal subsets (including subsets reactive to RFamide antisera and to the monoclonal antibody (Ab) JD1) and the presence of chemical and electrical synapses, action potentials, temporal and spatial summation, and miniature end-plate potentials (Roberts and Mackie, 1980; Grimmelikhuijzen *et al.*, 1980, 1982; Dunne *et al.*, 1985; Grimmelikhuijzen, 1985; Kerfoot *et al.*, 1985; Yu *et al.*, 1986; Fraser *et al.*, 1987; Anderson and Spencer, 1989; Green, 1989; Spencer, 1989; Umbriaco *et al.*, 1990; Sakaguchi *et al.*, 1996; Westfall, 1996). Thus, cnidarians are useful for studying basic nervous system phenomena such as development and synaptic transmission in isolation from the added complexity of large numbers of neurons. Hydra are a particularly good model in which to study nervous system development because they have a few easily observed morphologically and immunochemically defined neuron types.

Adult hydra continually develop and integrate numerous subsets of neurons. This ongoing development occurs by continual translocation of the neurons along the body length axis, which in turn is due to the continual translocation of the tissues in which the neurons are embedded (Campbell, 1966, 1974; Bode *et al.*, 1988a). Some of the neuronal subsets have been defined by their immunoreactivities (Grimmelikhuijzen, 1983a, 1984; Koizumi *et al.*, 1992). For instance, immunoreactivity to the neuropeptide FMRFamide is broadly distributed in cnidarians and has been detected in the head (in both the hypostome and tentacles), base, and body column of hydra (Grimmelikhuijzen, 1983b; Grimmelikhuijzen *et al.*, 1989). Antisera to the RFamide carboxyterminus of FMRFamide (Grimmelikhuijzen and Graff, 1985) yield a similar staining pattern. However, FMRFamide is not the only neuropeptide ending in RF-

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amide in hydra (Mitgutsch *et al.*, 1999). In adult *Hydra magnipapillata*, three separate genes code for four different carboxyterminus RFamides (Mitgutsch *et al.*, 1999). Although the RFamide genes are expressed in different subpopulations of neurons, RFamide immunoreactivity coincides with the total distribution of expression of the three genes (Grimmelikhuijzen and Graff, 1985; Mitgutsch *et al.*, 1999). Thus, RFamide Ab detects a family of RFamide-positive neurons.

Neurons identified by markers other than RFamide can show different staining and developmental patterns in adult hydra. The monoclonal Ab JD1 recognizes an uncharacterized antigen (Dunne *et al.*, 1985), and though the JD1 and RFamide Abs both recognize neurons, the distribution of their immunoreactivities is not identical. For instance, whereas RFamide antisera label neurons in the head, base, and body column in *Hydra vulgaris* (Grimmelikhuijzen, 1983b, 1985), JD1 stains neurons only in the head and base of *Hydra oligactis* (Dunne *et al.*, 1985). This may simply be an interspecific difference, however. Also, the hypostomal nerve ring of *H. oligactis* contains neurons that are both RFamide-positive and JD1-positive, but although all JD1-positive neurons are RFamide-positive, the opposite is not true (Koizumi *et al.*, 1992).

Though hydra are generally known as animals that reproduce by budding, some also reproduce sexually. Sexual reproduction in hydra involves gametogenesis that results in external eggs, spermaries, or both, and is followed by external fertilization, development of a spherical embryo (initially attached to the mother), addition of a protective cuticle, and hatching (Martin, 1997; Martin *et al.*, 1997). Cuticle deposition is complete 48 h after fertilization; the cuticle stage lasts between 2 and 24 weeks (Martin *et al.*, 1997). Two days before hatching, two distinct layers of cells appear in the embryo (Martin *et al.*, 1997). This subphase of the cuticle stage is termed the minus-2-d stage. At hatching, the hydra, which has not yet formed tentacles, is roughly cigar-shaped and is described as being in the cigar stage. Complete embryonic development can take as little as about 3 weeks or as long as about 25 weeks. The tentacle bud stage begins when tentacle formation is apparent—as early as 15 min after hatching—and lasts until the animal is 1 day old.

The architecture and development of the nervous system and the distribution of neuronal subpopulations have been extensively studied in the adult hydra but not in the embryonic animal. It is known that neurons appear 2 d prior to hatching in maceration studies of sequentially aged hydra embryos (Martin *et al.*, 1997). At that stage, RFamide-positive neuron clusters are found at both the head and base ends (Martin, 1997). A better, and quantitative, understanding of when neuron subpopulations arise and how their pool sizes change during and after embryogenesis is important because it will help frame questions about relationships

between neuronal subsets and about relationships between neuronal subsets and development itself.

The population dynamics of developing nervous systems throughout the animal kingdom display culling *via* programmed cell death (PCD) (Galliresta and Resta, 1992). Virtually all developing vertebrate nervous systems use this mechanism, which in many subpopulations is so prevalent that about half the neurons undergo PCD (Burek and Oppenheim, 1996; Sastry and Rao, 2000). PCD is also found in developing nervous systems of invertebrates. In the larval moth *Manduca sexta*, 10% to 70% of the neurons in the central nervous system (CNS) undergo PCD, and eclosion of the adult is followed by PCD of up to 50% of the CNS neurons (Booker *et al.*, 1996; Ewer *et al.*, 1998). Flies, nematodes, grasshoppers, and leeches also exhibit neuronal PCD (Stewart *et al.*, 1987; Shankland and Martindale, 1989; Robinow *et al.*, 1993; Sanders and Wride, 1995). Finally, PCD of peptidergic neurons has been observed in the fly and the moth (Ewer *et al.*, 1998; Draizen *et al.*, 1999). Apoptosis, a type of PCD, has recently been reported to result in the loss of the whole interstitial cell lineage in a hydra mutant (Cikala *et al.*, 1999). The same paper also identifies two enzymes belonging to the caspase 3 family of aspartate-specific cysteinyl proteases, and holds that both are apparently expressed in all hydra cell types (Cikala *et al.*, 1999). Because caspase activity is associated with apoptosis in nematodes, flies, and mammals (Cikala *et al.*, 1999), the presence of caspases in all hydra cell types suggests all these cells, including neurons, have the capacity to undergo apoptosis. Given that culling of developing nervous systems is widespread in the animal kingdom and that all hydra cells are apparently capable of PCD, it is interesting that PCD (or culling as an indicator of PCD) has not been reported for the developing nervous system of any part of the hydra's life history. Perhaps large-scale PCD occurs in hydra neuroembryogenesis, as it does in many other embryonic systems. If so, an indicator that this is occurring would be a significant decrease in neuron numbers at some point in nervous system development. Therefore, we characterized the development of the RFamide-positive and JD1-positive neuron subpopulations of hydra embryos, posthatching hydra, and adults to understand their population dynamics. This is the first study in which subpopulations of neurons in sexually derived hydra have been studied over developmental time.

## Materials and Methods

Stock cultures of *Hydra vulgaris* were maintained in "hydra" water (1 mM CaCl<sub>2</sub>, 1.5 mM NaHCO<sub>3</sub>, 0.1 mM MgCl<sub>2</sub>, 0.08 mM MgSO<sub>4</sub>, and 0.03 mM KNO<sub>3</sub> in double-distilled water, pH 7.0) at 18°C on a cycle of 12 h of light and 12 h of dark. The female hydra strain, designated PA2, was isolated from a pond on the Haverford College campus near Philadelphia, Pennsylvania. The male strain, CA7, was

collected from Boulder Creek, near Susanville, California. Embryos were obtained by placing spermary-carrying males with egg-carrying females. Embryos were cut from the mother with a surgical knife after the formation of the protective cuticle, placed in petri dishes of hydra water, and allowed to develop. Adult hydra were fed once a week with the brine shrimp *Artemia salina*, and the medium was changed twice a week. The following pre-adult stages were stained to characterize the temporal distribution of the JD1-positive and RFamide-positive neurons ( $n = 5$  for single staining,  $n = 1$  for double staining); embryos that had formed cuticles 10 d earlier (10-d cuticles), 20-d cuticles, minus-2-d cuticles, cigar stage, tentacle bud stage, and 5-d-old hatchlings. For the JD1 study, the adults stained were gameteless CA7 *H. vulgaris*. For the RFamide study, gameteless adult *H. vulgaris* from CA7/PA2 crosses were stained. This adult stock was created by allowing the hydra resulting from sexual crosses to bud, thus creating genetic clones of hatchlings. Adults were starved at least 48 h before fixing, and the younger animals were never fed.

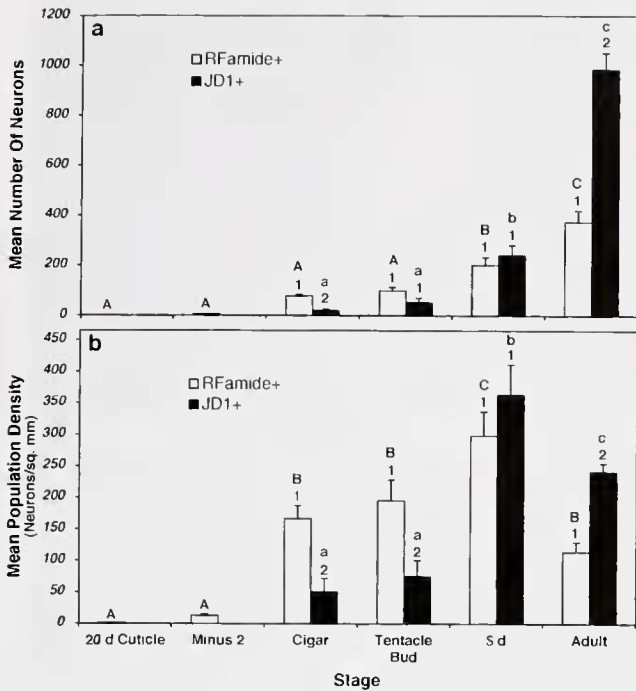
Wholemounts of the aforementioned developmental stages were stained for RFamide, the JD1 antigen, or dual (RFamide and JD1) immunoreactivity. Staining with the anti-RFamide polyclonal Ab was based on the procedure of Bode *et al.* (1988b), and staining with the monoclonal Ab JD1 was based on the procedure of Dunne *et al.* (1985). Because the protocols are very similar, they are presented together, with any variations noted. All cuticled stages were fixed in Zamboni's fixative (2.0% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.0) for the RFamide procedure or in Lavdowsky's fixative (50 units ethanol:10 formalin:4 acetic acid:40 ddH<sub>2</sub>O) for the JD1 procedure for 2–3 h, then placed in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.008 M Na<sub>2</sub>HPO<sub>4</sub>, and 0.002 M NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, pH 7.0), where the cuticles were surgically removed from the embryos with a scalpel. All post-hatching hydra were relaxed for 1–2 min in 2% urethane in hydra water. De-cuticled embryos were then further fixed, and posthatching relaxed hydra were fixed for the first time (overnight, 7 °C). After fixation, all animals were treated sequentially as follows (room temperature (21 °C) at 80 rpm on an orbital shaker unless otherwise noted): PBS-Tween (0.05% Tween 80 in PBS; 3 × 20 min for RFamide procedure, 3 × 10 min for JD1 procedure); 0.4 M glycine, pH 7.0 (1 h, RFamide procedure only); PBS-Triton (0.25% Triton X-100 in PBS; 3 × 20 min for RFamide procedure, 3 × 10 min for JD1 procedure); 10% fetal bovine serum (FBS) (in 0.1% NaN<sub>3</sub> in PBS, pH 7.0; overnight, 7 °C); primary antiserum (overnight, 7 °C; RFamide antiserum 146 III (Grimmelikhuijzen, 1985) diluted 1:1000 in 0.1 M PBS, pH 7.2, containing 0.05% Tween 80 and 0.25% human serum albumin or JD1 monoclonal Ab (Dunne *et al.*, 1985)); PBS-Triton (3 × 20 min); secondary Ab (in total dark as were all subsequent incubations, 1 h, FITC conjugated goat

anti-rabbit IgGs 1:60 in 10% FBS for RFamide procedure and FITC conjugated goat anti-mouse IgGs 1:50 in 10% FBS for JD1 procedure (Boehringer Mannheim)); PBS-Triton (3 × 20 min); and PBS (10 min). Wholemounts from cigar stage through adult were double-stained for RFamide and JD1 in a procedure similar to that of Koizumi *et al.* (1992). The first half of the procedure was the same as that for RFamide staining except that the primary Ab was JD1 and the secondary Ab was that used in the JD1 procedure. The second half of the double-staining procedure was the RFamide staining procedure (beginning with the incubation in RFamide antisera), except that the secondary Ab was rhodamine-conjugated goat anti-rabbit IgG and diluted 1:600. Controls for the single-staining procedures included omission of the primary Ab. Also, adult hydra were stained as controls with all younger stages in both the single- and double-staining procedures.

The positive-staining ganglion, sensory, and unclassifiable neurons were counted (JD1 also stained interstitial cells and nematoblasts, but only the neuron work is presented here). After staining, each hydra was placed in mounting medium (5% *n*-propyl gallate and 50% glycerin in PBS) and between two coverslips (the coverslip-hydra-coverslip sandwich was attached to the microscope slide by a drop of oil). Because each sandwich could be flipped over, both sides of a hydra were equally visible under the microscope, thus allowing the number of positive-staining neurons in each animal to be counted (Zeiss IV FL epi-fluorescence condenser, 450–490 nm and 546 nm filters). After the neurons in an animal were counted, the area of the body was measured with Universal Imaging's Image-1 software. Densities were then calculated for each neuron type at each stage. Sizes of each neuron population, densities of each population, and the sizes of the animal itself (in square millimeters) were compared among the developmental stages *via* one-way ANOVA and Tukey's honestly significant difference *post-hoc* test. Also, the total (ganglion cells + sensory cells + unclassifiable neurons) number of RFamide-positive neurons was compared to the total number of JD1-positive neurons, and the density of the total RFamide-positive population was compared to the density of the total JD1-positive population at each stage. These pairs of RFamide-positive and JD1-positive total neuron numbers or total neuron population densities within a stage were compared *via t* tests run as independent and not assuming equal variances. Differences were considered significant at  $P \leq 0.05$ .

## Results

The total number of RFamide-positive neurons and the total number of JD1-positive neurons increased throughout the hydras' development (Fig. 1a). In contrast, the total densities of each population increased, then decreased (Fig.



**Figure 1.** Mean ( $\pm$  standard error of the mean (SEM)) total numbers (a) and population densities (b) of RFamide-immunoreactive neurons and JD1-immunoreactive neurons in different developmental stages of *Hydra vulgaris* ( $n = 5$ ). One-way ANOVA for RFamide-positive neurons across all stages and for JD1-positive neurons across the last four stages showed significant differences (for number of RFamide-positive neurons  $F = 36.75$ ,  $df = 5$ ,  $P = 0.001$ ; for number of JD1-positive neurons  $F = 129.39$ ,  $df = 3$ ,  $P = 0.001$ ; for density of RFamide-positive neuron population  $F = 23.67$ ,  $df = 5$ ,  $P = 0.001$ ; for density of JD1-positive neuron population  $F = 24.52$ ,  $df = 3$ ,  $P = 0.001$ ). Results of *post-hoc* Tukey's honestly significant difference tests are indicated with letters (uppercase for RFamide results and lower case for JD1 results). Within-stage differences between the RFamide-positive population and the JD1-positive population were compared for each of the last four stages *via t* tests. The cigar stage (separate variances  $t = 8.79$ ,  $df = 8$ , and  $P = 0.001$ ) and adult (separate variances  $t = -7.61$ ,  $df = 7$ ,  $P = 0.001$ ) each showed a significant difference between the total RFamide-positive population and the total JD1-positive neuron population. The cigar stage (separate variances  $t = 3$ ,  $df = 8$ , and  $P = 0.001$ ), tentacle bud stage (separate variances  $t = 2.88$ ,  $df = 7$ , and  $P = 0.02$ ), and adult (separate variances  $t = -6.27$ ,  $df = 8$ , and  $P = 0.001$ ) also each showed a significant difference between the two neuron population densities. Results of the *t* test are indicated by numbers. Bars that share a letter or number within the range of an ANOVA or *t* test are not significantly different ( $P > 0.05$ ).

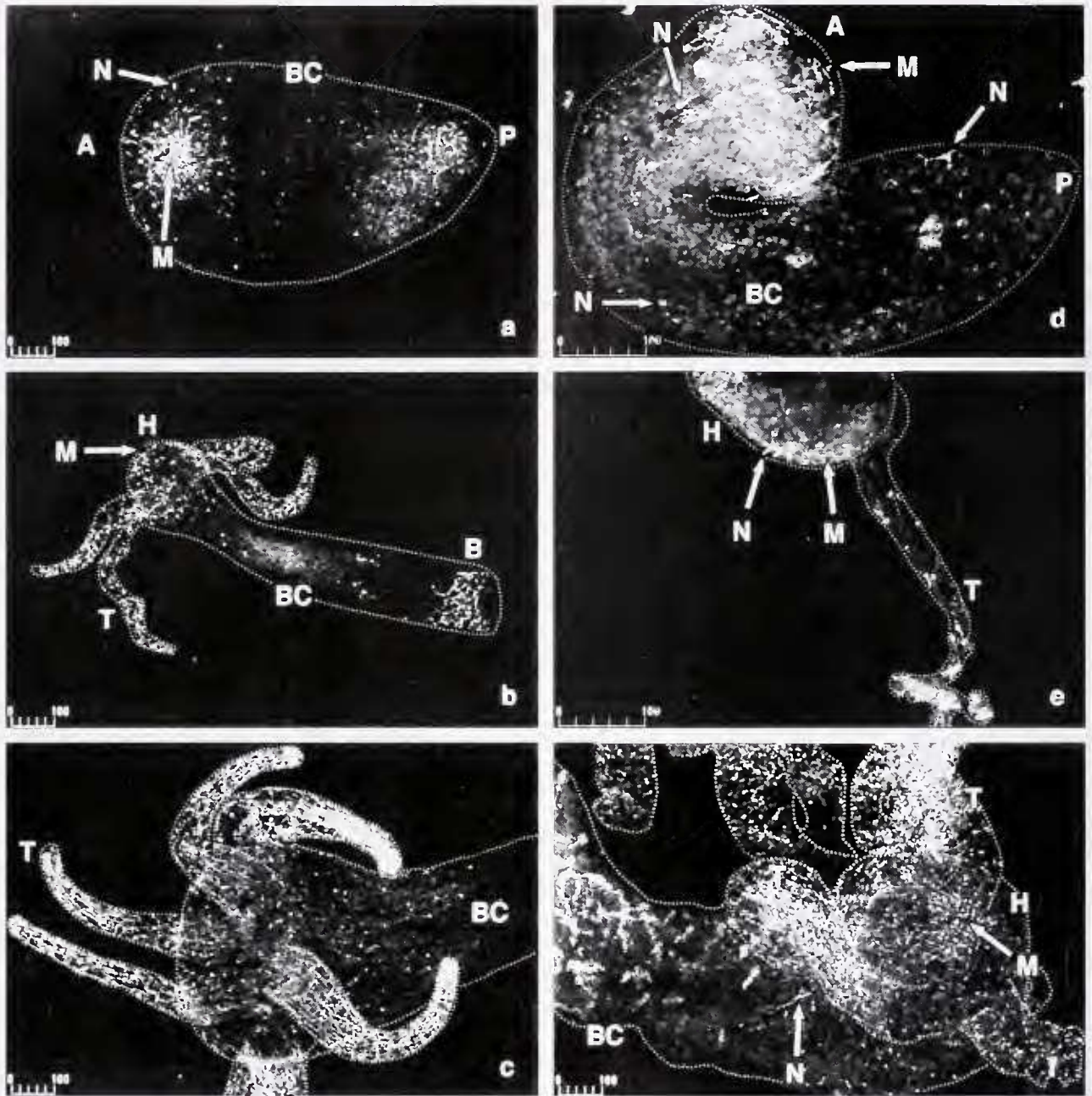
1b). All significant changes in the number of RFamide-positive neurons and in the number of JD1-positive neurons were increases (Fig. 1a). The density of each population declined significantly after the 5-d stage (Fig. 1b). The RFamide-positive population was initially more numerous and dense than the JD1-positive population, but the ratios later reversed such that the JD1-positive population was more numerous and dense (Fig. 1). In the cigar stage, the numbers of RFamide-positive neurons exceeded those of JD1-positive neurons significantly, but the adult stage

showed the opposite with JD1-positive neurons significantly more prevalent. Similarly, in the cigar stage the RFamide-positive population was significantly more dense than the JD1-positive neuron population but the adult showed the opposite result.

RFamide-positive neurons were not present in the 10-d cuticle stage but were in every stage examined thereafter. They were first seen in some of the 20-d cuticle stage hydra, and in later stages both the number of RFamide-positive neurons and the complexity of the RFamide-positive nerve net increased (Figs. 1, 2a–c). However, the size of the RFamide-stained hydra did not increase until after the 5-d stage. Areas of the pre-adult stages in which RFamide-positive neurons were found ranged from  $0.3 \text{ mm}^2$  ( $\pm 0.03 \text{ mm}^2$  standard error of the mean (SEM)) for the 20-d cuticle to  $0.7 \text{ mm}^2$  ( $\pm 0.1 \text{ mm}^2$  SEM) for the 5-d and did not differ significantly. The increase from 5 d to adult ( $3.5 \text{ mm}^2 \pm 0.6 \text{ mm}^2$  SEM) was significant ( $P < 0.001$ ). In contrast to the RFamide-positive neurons, JD1-positive neurons first appeared in the cigar stage. However, like the RFamide-positive neuron population, once the JD1-positive population arose, both the number of JD1-positive neurons and the complexity of the JD1-positive nerve net increased (Figs. 1, 2d–f). Pre-adult hydra stained for JD1 did not differ in area, but the adults stained for JD1 were significantly larger than the 5-d stage ( $P < 0.001$ ).

The population trends of RFamide-positive neuron subtypes reflected the trends of total RFamide-positive neuron numbers and densities (Fig. 3). Like the RFamide-positive neuron population as a whole, all significant changes in the RFamide-positive ganglion cell, RFamide-positive sensory cell, and RFamide-positive unclassifiable neuron numbers between stages were increases (Fig. 3a). Furthermore, the population density of the RFamide-positive ganglion cells declined significantly after the 5-d stage (Fig. 3b), as was seen in the density dynamics of the whole RFamide-positive neuron population. Finally, neither the mean population density of the RFamide-positive sensory cells nor that of the RFamide-positive unclassifiable neurons decreased significantly after the 5-d stage. However, the shape of the graphs of each of these populations over time is reminiscent of the density dynamics of the whole RFamide-positive neuron population (Figs. 1b, 3b).

The population trends of the JD1-positive neuron subtypes reflected the trends of total JD1-positive neuron numbers and densities (Fig. 4). All significant changes in JD1-positive ganglion cell, JD1-positive sensory cell, and JD1-positive unclassifiable neuron numbers between stages were increases (Fig. 4a), mirroring the population dynamics of the JD1-positive neuron population as a whole. Also, there were significant increases in the population densities of JD1-positive ganglion cells, JD1-positive sensory cells, and JD1-positive unclassifiable neurons by the 5-d stage (Fig. 4b). But the JD1-positive ganglion cell and JD1-positive

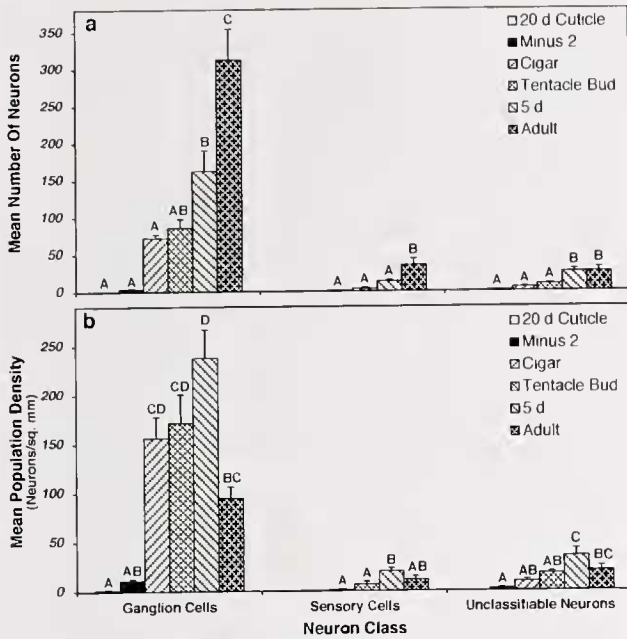


**Figure 2.** Single-plane confocal images of RFamide-positive and JD1-positive nerve net development. A, anterior; P, posterior; H, hypostome; M, mouth; T, tentacle; BC, body column; B, base; N, individual neuron. Scale bars are in  $\mu\text{m}$ . (a, b, c) The left column indicates the increasing numbers of neurons and complexity of the RFamide-positive nerve net as it progressed from the cigar stage (a) to the 5-d stage (b) to the adult (c, only shows upper body column and head). (d, e, f) The right column indicates the increasing numbers of neurons and complexity of the JD1-positive nerve net as it progressed from the cigar (d) to the 5-d stage (e, only shows head) to the adult (f, only shows upper body column and head).

unclassifiable neuron population densities both decreased significantly after the 5-d stage (Fig. 4b), reflecting the density dynamics of the total JD1-positive neuron population. Finally, the mean JD1-positive sensory cell population density did not decrease significantly after the 5-d stage, but

the shape of the graph of this population over time is reminiscent of the density dynamics of the whole JD1-positive population (Figs. 1b, 4b).

The RFamide staining protocol labeled a population of neurons largely distinct from that labeled by the JD1 stain-



**Figure 3.** Mean ( $\pm$  SEM) numbers (a) and population densities (b) of RFamide-positive ganglion cells, sensory cells, and unclassifiable neurons in different developmental stages of *Hydra vulgaris* ( $n = 5$ ). One-way ANOVA for numbers and population densities of ganglion cells across all six stages studied, sensory cells across the last four stages, and unclassifiable neurons across the last five stages showed significant differences (RFamide-positive ganglion cell number,  $F = 31.73$ ,  $df = 5$ ,  $P = 0.001$ ; density of RFamide-positive ganglion cell population,  $F = 23.02$ ,  $df = 5$ ,  $P = 0.0001$ ; RFamide-positive sensory cell number,  $F = 13.02$ ,  $df = 3$ ,  $P = 0.001$ ; density of RFamide-positive sensory cell population,  $F = 8.77$ ,  $df = 3$ ,  $P = 0.001$ ; RFamide-positive unclassifiable neuron number,  $F = 15.94$ ,  $df = 4$ ,  $P = 0.001$ ; density of RFamide-positive unclassifiable neuron population,  $F = 8.76$ ,  $df = 4$ ,  $P = 0.001$ ). Results of *post-hoc* Tukey's honestly significant differences test are indicated with letters—bars sharing a letter within an ANOVA series are not significantly different ( $P > 0.05$ ).

ing protocol. Though some double labeling was observed in each of the posthatching stages ( $n = 1$  for double staining), the numbers of double-labeled neurons were minimal compared to the average total RFamide-positive and JDI-positive neuron populations found in the single-stained samples ( $n = 5$ ) of those stages. The percentage of double-labeled neurons ranged from a low of 1% of RFamide-positive neurons and 1% of JDI-positive neurons in the 5-d stage to a high of 5% of RFamide-positive neurons and 10% of JDI-positive neurons in the tentacle bud stage.

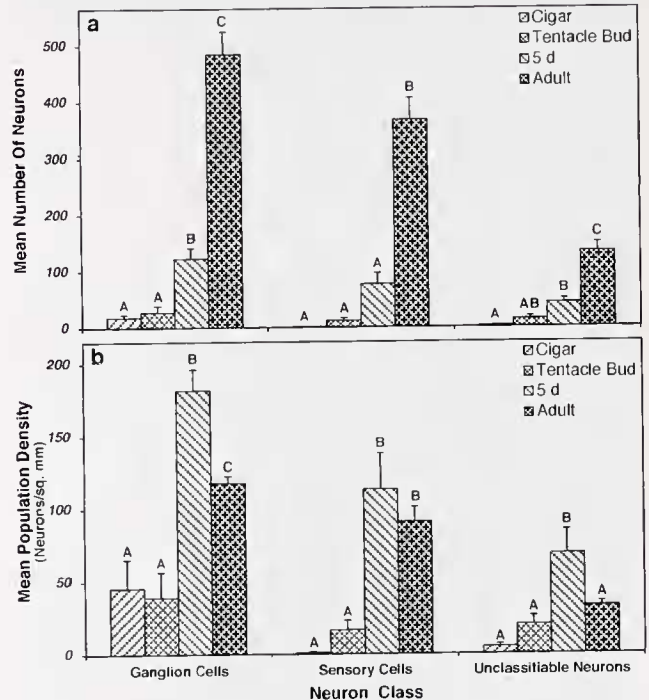
Adult hydra stained *via* the RFamide or JDI procedures minus their respective primary Abs displayed no stained cells (Fig. 5). Also, positive-staining adults were present as controls in every assay that produced nonstaining embryos.

### Discussion

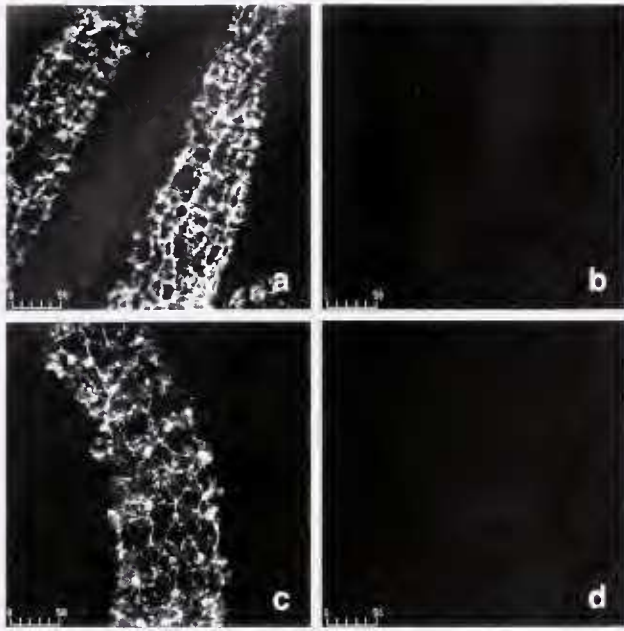
This study provides evidence based on neuron counts over developmental time that large-scale programmed cell

death might not occur in the RFamide-positive or JDI-positive neuron populations of developing hydra. Similar evidence is provided that large-scale PCD might not occur during the development of the RFamide-positive or JDI-positive subpopulations (ganglion, sensory, or unclassifiable). If massive neuron culling were occurring in these populations, then their neuron counts would decrease (excepting the possibility that PCD and neurogenesis are concurrent but that neurogenesis outpaces PCD). The results showed that the neuron counts did not decrease during (or after) embryogenesis (Figs. 1, 3, 4). Therefore, culling *via* PCD might not occur on a large scale in these neuron populations during or after hydra embryogenesis.

This hypothesis is consistent with whole-neuron counts in two other modes of hydra development—budding and head regeneration. In five stages of bud development the total neuron number progresses as follows: 350, 500, 750, 1500,



**Figure 4.** Mean ( $\pm$  SEM) numbers (a) and population densities (b) of JDI-positive ganglion cells, sensory cells, and unclassifiable neurons in different developmental stages of *Hydra vulgaris* ( $n = 5$ ). One-way ANOVA for numbers and population densities of ganglion cells, sensory cells, and unclassifiable neurons across the JDI-positive stages showed significant differences (JDI-positive ganglion cell number,  $F = 88.96$ ,  $df = 3$ ,  $P = 0.001$ ; density of JDI-positive ganglion cell population,  $F = 19.45$ ,  $df = 3$ ,  $P = 0.001$ ; JDI-positive sensory cell number,  $F = 61.08$ ,  $df = 3$ ,  $P = 0.001$ ; density of JDI-positive sensory cell population,  $F = 15.87$ ,  $df = 3$ ,  $P = 0.001$ ; JDI-positive unclassifiable neuron number,  $F = 46.52$ ,  $df = 3$ ,  $P = 0.001$ ; density of JDI-positive unclassifiable neuron population,  $F = 9.39$ ,  $df = 3$ ,  $P = 0.001$ ). Results of *post-hoc* Tukey's honestly significant differences test are indicated with letters—bars sharing a letter within an ANOVA series are not significantly different ( $P > 0.05$ ).



**Figure 5.** Single-plane confocal images of controls. Scale bars are in  $\mu\text{m}$ . (a, b) Tentacles processed with the RFamide antibody revealed a nerve net (a), whereas tentacles processed without the RFamide antibody did not (b). (c, d) Tentacles stained with the JD1 antibody revealed a nerve net (c), whereas tentacles processed using the same protocol minus the JD1 antibody did not (d).

2500 (Bode *et al.*, 1973). The authors report 5600 neurons in the adult. Similarly, regenerating heads show no decreases in neuron number. A decapitated hydra generates its new head from the tissue below the old head (Bode *et al.*, 1988a). Initially about 250 neurons populate the subhead region, and this number increases to 300, 500, and 700 as this tissue becomes head; the adult head contains about 800 neurons (Bode *et al.*, 1973). Thus, in embryological development, budding, and head regeneration, the hydra neuron populations studied show no culling. This indicates that continuous increase in neuron numbers through adult levels may be a common mechanism of neurodevelopment in hydra.

The hypothesis that large-scale neuronal PCD might not be part of adult hydra development is also supported by studies of tissue dynamics. First, loss of tissue to buds and to sloughing at the extremities balances the constant neuron production occurring in the adult hydra (David and Gierer, 1974; Bode *et al.*, 1988a; Sakaguchi *et al.*, 1996). If massive neuronal culling were occurring in the constantly developing adult, then neuron loss through budding and sloughing would not balance neuron production. However, calculations suggest that these two activities do balance neuron production (David and Gierer, 1974; Bode *et al.*, 1988a). This leaves little room for large-scale PCD of neurons between neurogenesis and sloughing. Second, if many neurons died between genesis and sloughing in adults, then neurons would be less long-lived than if they made the

whole journey. However, quantitative studies of the whole neuron populations of the hypostome and the region of the body column apical to the budding zone (the gastric region) indicate that neurons in these regions live at least several times the mass-doubling period of an adult hydra's tissue and perhaps live indefinitely (David and Gierer, 1974). Thus, the lack of neuron population decrease in embryos, buds, and regenerating heads is consistent with the finding of David and Gierer (1974) that neurons of adult hydra are long-lived in the hypostome and gastric region. David and Gierer's results also suggest neurons live long enough to reach an extremity and be sloughed or to reach a bud. So, the normal tissue dynamics of the adult hydra leave very little room for large-scale PCD of neurons and are therefore consistent with the hypothesis that neuronal culling does not occur during embryogenesis.

The combination of neuron counts and studies of tissue dynamics outlined above indicates that massive neuron death might not occur during the development of the hydra nervous system, though it does in many more advanced nervous systems. Individual neurons and synapses of enidarian nervous systems exhibit basic characteristics like those of more evolved systems (Roberts and Mackie, 1980; Kerfoot *et al.*, 1985; Fraser *et al.*, 1987; Anderson and Spencer, 1989; Grimmelikhuijzen and Westfall, 1995; Westfall, 1996). But greater numbers of neurons, more complex architectures, more specialized neurons, and a lesser emphasis on peptides appeared in nervous systems later in evolution (Grimmelikhuijzen and Westfall, 1995; Grimmelikhuijzen *et al.*, 1996). Large-scale culling of neurons during development may also have evolved later.

However, it appears hydra cells, including neurons, actually do come fully equipped with apoptotic machinery (Cikala *et al.*, 1999). This apoptotic machinery in hydra neurons may be used for any number of purposes other than culling neurons overproduced during development. PCD operates in contexts such as responding to DNA damage, defending against viruses and cancer, splitting of tissues in development, and shedding old tissue (Shikone *et al.*, 1997; Ahmad and Golic, 1999; Mire and Venable, 1999; Simon *et al.*, 1999; Gartner *et al.*, 2000; Ollmann *et al.*, 2000; Ramachandran *et al.*, 2000; Sastry and Rao, 2000). More specifically, PCD facilitates fission in sea anemones (Mire and Venable, 1999); perhaps it has a similar function during budding in hydra. Finally, PCD is important in the shedding of mammalian endometrial and intestinal epithelia (Shikone *et al.*, 1997; Simon *et al.*, 1999; Ramachandran *et al.*, 2000); perhaps it is similarly important in the continual sloughing of hydra cells (including neurons).

It may be possible to explain the apparent constant increase in hydra neuron numbers and also invoke massive culling of neuron populations that could not be detected by neuron counts. An organism as plastic as the hydra could be undergoing the massive neuroapoptosis associated with

other model systems but still produce enough new neurons to mask culling. A study of cell-death markers during development of the hydra nervous system is required to examine this possibility.

Differentiation and maintenance of neuron populations in hydra does not keep pace with the animal's growth in size. If it did, neuron densities would remain constant throughout development. However, most of the neuron population densities studied in the sexually derived hydra decreased significantly (Figs. 1, 3, 4), failing to keep up with the 5-fold increase in size the present study has shown to occur after the 5-d stage. Similarly, in asexual reproduction, neurons compose 6.5% of the total cell number of a recently dropped *H. vulgaris* bud, but only 4.5% of an adult's total cell number (Bode *et al.*, 1973). As the bud adds mass after being dropped, this decrease in the percentage of neurons probably also represents a decrease in the density of neurons (measured as number per unit of tissue). So, RFamide-positive neurons and JD1-positive neurons of sexually derived hydra and, probably, the whole neuron population of buds differentiate less quickly relative to growth in size after the 5-d stage or bud dropping. This indicates that a mechanism common to both hydra embryogenesis and budding may be involved in regulating neuronal density.

Increased feeding of adult hydra moderately increases the proportion of epithelial cells to neurons (Bode *et al.*, 1973). The same may apply to buds and young sexually derived hydra and would explain why their neuron populations differentiate less quickly relative to growth in size after either the 5-d stage or bud dropping. In the present study, no developmental stages were fed. But, by the 2-d stage, hydra have both nematocytes mounted on tentacles and tentacles long enough to function in feeding (Martin *et al.*, 1997). Sexually derived hydra in the wild, or in the laboratory if allowed, increase their food intake after this stage. Likewise, buds may increase their food consumption as their cell-type distribution and morphology mature. As maturing buds and sexually derived hydra eat more, the proportion of epithelial cells to neurons will increase moderately, assuming that the cell-type proportions are regulated as in the adult. If neurons and epithelial cells were the same size, then this increase in the proportion of epithelial cells to neurons would moderately decrease neuron density measured as number of neurons per unit of tissue. However, because epithelial cells are much larger than neurons, a moderate increase in the number of epithelial cells to the number of neurons may be magnified into a significant decrease in the density of neurons per unit area (as seen in maturing sexually derived hydra). Perhaps neurite arborization is increased during this time of rapid growth and decreasing neuron density to maintain connectivity to the increasing bulk of tissue.

RFamide-positive neurons arose much earlier than JD1-positive neurons and, when the two types were present

concurrently, were greater in number and density in early stages than JD1-positive neurons (Fig. 1). These proportions reversed in later stages. RFamide-positive neurons appear 1/2 d to 2 d prior to the first signs of head regeneration in adult *H. vulgaris* (Bode *et al.*, 1988b; Koizumi *et al.*, 1990), and the RFamide-positive neuron network already exists at the future head end of the minus-2 stage (Martin, 1997), 2 d before tentacles arise. Thus, RFamide-positive neurons appear before head formation in the regenerating adult and in the embryo. On the other hand, studies of *H. oligactis* indicate that JD1-positive neurons may appear concurrently with head structures in both budding hydra and hydra regenerating heads (Dunne *et al.*, 1985). In the embryo, JD1-positive neurons were first seen in the cigar stage. If, indeed, those neurons did appear at or near hatching, then neurogenesis of JD1-positive neurons correlates with the appearance of the embryo's head morphology, because tentacle buds form within a few minutes of hatching. Thus, the sequence of differentiation of RFamide-positive and JD1-positive neurons might occur with similar timing in embryos, buds, and regenerating heads.

In summary, the neuron subpopulations quantified in this study show no evidence of large-scale PCD during or after hydra embryogenesis; neither do neuron counts nor studies of tissue dynamics in budding, head regeneration, or the constantly developing adult hydra. Neuron population densities decrease after reaching a maximum in both embryonic and bud development, and the sequence of RFamide-positive and JD1-positive neurogenesis might be the same in embryos, buds, and regenerating heads. These commonalities indicate that neurodevelopment strategy may be highly conserved among the various modes of hydra development.

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